Astrovirus MLB2, a New Gastroenteric Virus Associated with Meningitis and Disseminated Infection

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Next-generation sequencing has identified novel astroviruses for which a pathogenic role is not clearly defined. We identified astrovirus MLB2 infection in an immunocompetent case-patient and an immunocompromised patient who experienced diverse clinical manifestations, notably, meningitis and disseminated infection. The initial case-patient was identified by next-generation sequencing, which revealed astrovirus MLB2 RNA in cerebrospinal fluid, plasma, urine, and anal swab specimens. We then used specific real-time reverse transcription PCR to screen 943 fecal and 424 cerebrospinal fluid samples from hospitalized patients and identified a second case of meningitis, with positive results for the agent in the patient's feces and plasma. This screening revealed 5 additional positive fecal samples: 1 from an infant with acute diarrhea and 4 from children who had received transplants. Our findings demonstrate that astrovirus MLB2, which is highly prevalent in feces, can disseminate outside the digestive tract and is an unrecognized cause of central nervous system infection.

Astroviruses, family *Astroviridae*, are small, nonenveloped, single-stranded RNA viruses. The family comprises 2 genera: *Mamastrovirus* species infect mammals, including humans, and *Avastrovirus* species infect poultry and other birds. Human astroviruses (HAstVs) were first identified in 1975 (*1*); until recently, only classic HAstVs that belonged to the species *Mamastrovirus* (MAstV) *1* were recognized as human pathogens. HAstVs contribute to ≈10% of nonbacterial, sporadic gastroenteritis in children, with the highest prevalence observed in community healthcare centers (*2*,*3*). Symptoms are generally mild, with patient hospitalization usually not required; asymptomatic carriage has been described in 2% of children (*4*).

Screening of fecal samples from persons with diarrhea and control samples in different parts of the world by unbiased next-generation sequencing (NGS) or reverse transcription PCR (RT-PCR) has revealed the sporadic presence of members of the *Astroviridae* family, previously unrecognized in humans, that are phylogenetically substantially distant from classic HAstVs (*3*,*5*–*9*). These viruses have been named HAstV-VA/HMO and HAstV-MLB, for Virginia, human-mink-ovine, and Melbourne, respectively, according to the place where they were first identified and their close phylogenetic distance to animal astroviruses; these viruses belong to distinct species (*10*).

Cellular receptors and targeted cells for these viruses are unknown and, to date, novel astroviruses have not been culturable. Although the primary site of astroviral replication seems to be the gastrointestinal tract, disseminated diseases and encephalitis have been associated with infection with classic and nonclassic astroviruses (*11*–*16*). In animals, astroviruses also have the potential to target other organs; hepatitis and nephritis have been observed in avian infections (*4*,*17*).

These observations point to the noteworthy genetic diversity of astroviruses and their probable cross-species transmission. Nonetheless, clinical disease associated with new astrovirus variants remains to be confirmed (*9*,*18*). Although HAstV-MLB has been recovered from fecal samples of patients with acute flaccid paralysis (*6*), to our knowledge, no reports have documented this variant in cerebrospinal fluid (CSF) or central nervous system (CNS) tissue samples.

In June 2013, we launched a single-center prospective study using NGS to determine potential viral etiologic agents of meningoencephalitic and respiratory syndromes.

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Yet, in ≈50% of meningoencephalitis cases clinically suspected to be of viral origin, origins remain undetermined, despite comprehensive microbiologic investigations (*19*,*20*). We report the detection, in the context of this project, of an astrovirus MLB2 in the CSF of an immunocompetent adult patient with acute meningitis who was hospitalized at the University of Geneva Hospitals, Geneva, Switzerland, and the results of the pilot prevalence study and clinical investigation that this discovery triggered.

Materials and Methods

Virus Discovery Study

The virus discovery study and the pilot retrospective prevalence study it generated were approved by the University of Geneva Hospitals (CCER no. 13-075), and informed consent was obtained from the case-patient. This single-center epidemiologic study is ongoing (online Technical Appendix 1, http://wwwnc.cdc.gov/EID/article/22/5/15-1807-Techapp1.pdf).

High-Throughput Sequencing and Sequence Analysis

High-throughput sequencing (RNA-seq library preparation, paired-end sequencing by using the 100-bp protocol with indexing on a HiSeq 2500 [Illumina, San Diego, CA, USA]) was performed directly on the case-patient's CSF, plasma, urine, and anal swab specimen, and we analyzed results using the ezVIR pipeline as described (*21*). Of note, a DNA-seq library was also prepared and analyzed for the screening of CSF specimens of the virus discovery study.

We used high-throughput sequencing data from the anal swab specimen to obtain a MLB2 consensus sequence by aligning the reads from the ezVIR output with those of the MLB2 genome Bowtie2 (*22*) and then assembling them using Sparse Assembler (*23*). We used the full sequence (GenBank accession no. KT224358) and the capsid region (corresponding to nt 3831–6069 on the consensus sequence and nt 3843–6080 on the reference sequence) to perform a phylogenetic analysis. We made multiple alignments using multiple alignment with the fast Fourier transform (*24*) and built the tree using IQTree (*25*), with 10,000 bootstrap replicates. The tree was created with Evolview (*26*) using reference strains from GenBank (online Technical Appendix 2 Tables 1, 2, http://wwwnc.cdc.gov/EID/article/22/5/15-1807-Techapp2.pdf).

Extraction and Construction of Specific Real-Time RT-PCR

We spiked 190-μL patient specimens of CSF, plasma, urine, anal swab, and nasopharyngeal aspirates with 10 μL of standardized canine distemper virus of known concentration (*27*) and extracted RNA with the NucliSENS easyMAG (bioMérieux, Geneva, Switzerland) nucleic acid kit in an elution volume of 25 μL, according to the manufacturer's instructions. We directly used extracted RNA for astrovirus MLB2–specific real-time RT-PCR screening analysis using an assay described by Holtz et al. (*11*). We performed PCR assay reaction using the QuantiTect Probe RT-PCR Kit (QIAGEN, Valencia, CA, USA) on a StepOnePlus instrument (Applied Biosystems, Rotkreuz, Switzerland) under the following cycling conditions: 50°C for 30 min, 95°C for 15 min, 45 cycles of 15 s at 94°C, and 1 min at 55°C. Data were analyzed with the StepOne software V.2 (Applied Biosystems). Analytical sensitivity was assessed with a plasmid including the target region and showed a limit of detection corresponding to 25 copies/reaction. We further analyzed positive specimens for confirmation with a second real-time RT-PCR targeting the viral RNA-dependent RNA polymerase region (forward primer 5′-TCCCTTCTGGTGAGGTCACTCT-3′, reverse primer 5′-AGGCTTGCAACCAATAGTTAATCAT-3′, and probe 5′-FAM-AACCGTGGTAATCCATCCGGTCAAATAT-CA-TAMRA-3′) under the following cycling conditions: 50°C for 30 min, 95°C for 15 min, 45 cycles of 15 s at 94°C, and 1 min at 60°C.

Pilot Prevalence Study

To estimate the local prevalence of this novel astrovirus, we tested some CSF specimens, including all of those with a total leukocyte count of >5 cells/μL, collected from April 2013 through April 2015, and all fecal specimens collected from August 2014 through August 2015 with the astrovirus MLB2–specific real-time RT-PCR targeting the capsid gene. Specimens were collected from pediatric and adult patients hospitalized at the University of Geneva Hospitals, a 1,900-bed tertiary-care medical center, and sent to the center's laboratory of virology for any clinical purpose. All samples had been stored at –80°C.

Results

Astrovirus MLB2 in Case-Patient

The CSF collected from a patient with acute meningitis who was enrolled in the virus discovery study tested positive for astrovirus MLB2 (Figure 1) with a total of 155 specific reads (35% genome coverage; total covered, 2,183 bp). Reads did not map to other RNA viruses, and DNA sequencing revealed no reads for bacterial or viral pathogens. For this case-patient, astrovirus MLB2–specific reads were further detected by NGS in the following acute-phase specimens: anal swab (70,890 reads, 9,340 after duplicate removal; 99% genome coverage; total covered, 6,107 bp); plasma (18 reads, 5% genome coverage; total covered, 336 bp); and urine (16 reads; 1% genome coverage; total covered, 120 bp) (Figure 2, panel A).

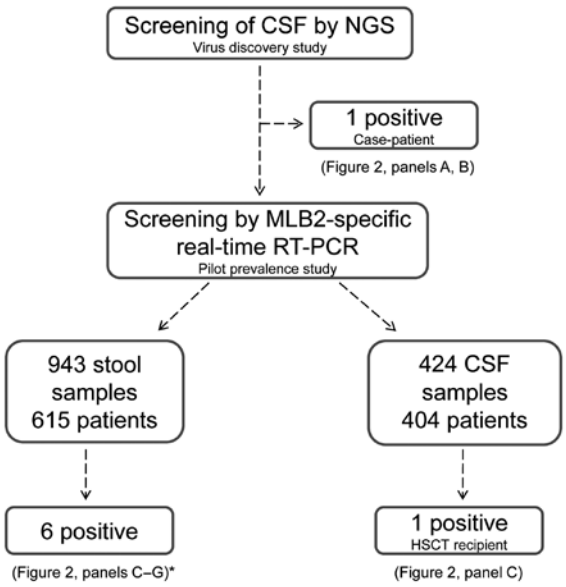

Figure 1. Flowchart of study using NGS to determine potential viral etiologic agents of meningoencephalitic and respiratory syndromes, Geneva, Switzerland, 2014. *The diarrheic immunocompetent infant is not represented in Figure 2. CSF, cerebrospinal fluid; NGS, next-generation sequencing; RT-PCR, reverse transcription PCR.

CSF obtained at hospital admission was confirmed positive by astrovirus MLB2–specific real-time RT-PCR targeting the capsid gene (*11*). Anal swab and urine specimens collected during the acute phase were also confirmed positive by astrovirus MLB2–specific RT-PCR, with the highest viral load found in the anal swab specimen. The plasma specimen drawn at admission showed a low viremia level, whereas plasma and additional CSF collected during the convalescent phase 5 and 2 days later, respectively, were negative (Figure 2, panel B). A second confirmatory assay targeting the RNA-dependent RNA polymerase gene confirmed all positive results (data not shown). Plasma and fecal specimens collected from the patient 8 months later were negative (Figure 2, panel B).

Phylogenetic analysis was performed on the full-length genome and on capsid sequences (Figure 3; online Technical Appendix 2 Figure, Table 1). Astrovirus MLB2 Geneva 2014 shows 98.5% nucleotide sequence identity homology with the complete genome of an astrovirus MLB2 isolate MLB2/human/Stl/WD0559/2008 detected in a viremic child in St. Louis, Missouri, USA, in 2011 (*11*).

Pilot Prevalence Study

We screened 943 fecal specimens from 615 unique patients; specimens from 6 patients (1%) were positive for astrovirus MLB2 by the 2 RT-PCR assays, bringing the overall number of positive patients to 7. Except for 1 immunocompetent infant who was brought for treatment with diarrhea of 15 days' duration, all patients were highly immunocompromised: 1 was an adult recipient of a hematopoietic stem cell transplant (HSCT) (Figure 2, panel C) and 4 were children who received solid organ transplants (Figure 2, panels D–G). Two patients had concomitant viremia (Figure 2, panels C and D), and 1 had 2 astrovirus MLB2–positive fecal samples, collected 2 weeks apart (Figure 2, panel G). Most patients had past or current digestive tract symptoms; the immunocompetent infant with diarrhea had no other digestive pathogen retrieved, and no other explanation was found for his symptoms. One child who had received a transplant experienced concomitant and recurrent *Clostridium difficile* infection, and adenovirus DNA was found in his feces.

Among 424 CSF specimens collected from 404 patients hospitalized in the 2 previous years, 1 supplementary specimen was positive for astrovirus MLB2. The patient was the HSCT recipient whose feces had also been screened positive (Figure 2, panel C). Of note, we detected astrovirus MLB2 RNA in CSF from this patient over a 3-month period and intermittently in plasma specimens from this patient over a 2-month period.

Meningitis: Clinical Case Descriptions

The case-patient (Figure 2, panels A, B) had been enrolled in the virus discovery study. In September 2014, this previously healthy 21-year-old woman sought treatment for an unusually severe headache and fever of a few hours' duration. She lived in a rural area, had 2 housecats, and had recently traveled to Portugal. She worked at a children's daycare center. Physical examination revealed neck stiffness without focal neurologic deficits. Blood laboratory test results were within reference limits; leukocyte count was 4.2×10^9 cells/L, and C-reactive protein level was 27 nmol/L. Analysis of CSF obtained by lumbar puncture (LP) at admission revealed clear fluid yet an abnormally high leukocyte count of 915 cells/μL (reference range 0–5 cells/mL), with 93% neutrophils; slightly elevated protein (73 mg/dL, reference range 15–45 mg/dL); and a CSF/plasma glucose ratio of 0.53. The patient was admitted and ceftriaxone and acyclovir were administered empirically. Bacterial CSF cultures remained negative, as did viral real-time RT-PCR assays targeting herpes simplex virus, varicella-zoster virus, enteroviruses, parechovirus, and Toscana virus. Serologic testing for HIV, *Treponema pallidum* (syphilis), tick-borne encephalitis virus, and *Borrelia burgdorferi* (Lyme disease) were negative, as were blood cultures. The patient underwent repeat LP 4 days after admission; the CSF leukocyte count had decreased to 47 cells/μL with a shift toward lymphocytic predominance (92%), whereas protein levels had returned to reference range (21 mg/dL). Real-time PCR results for herpes simplex virus and

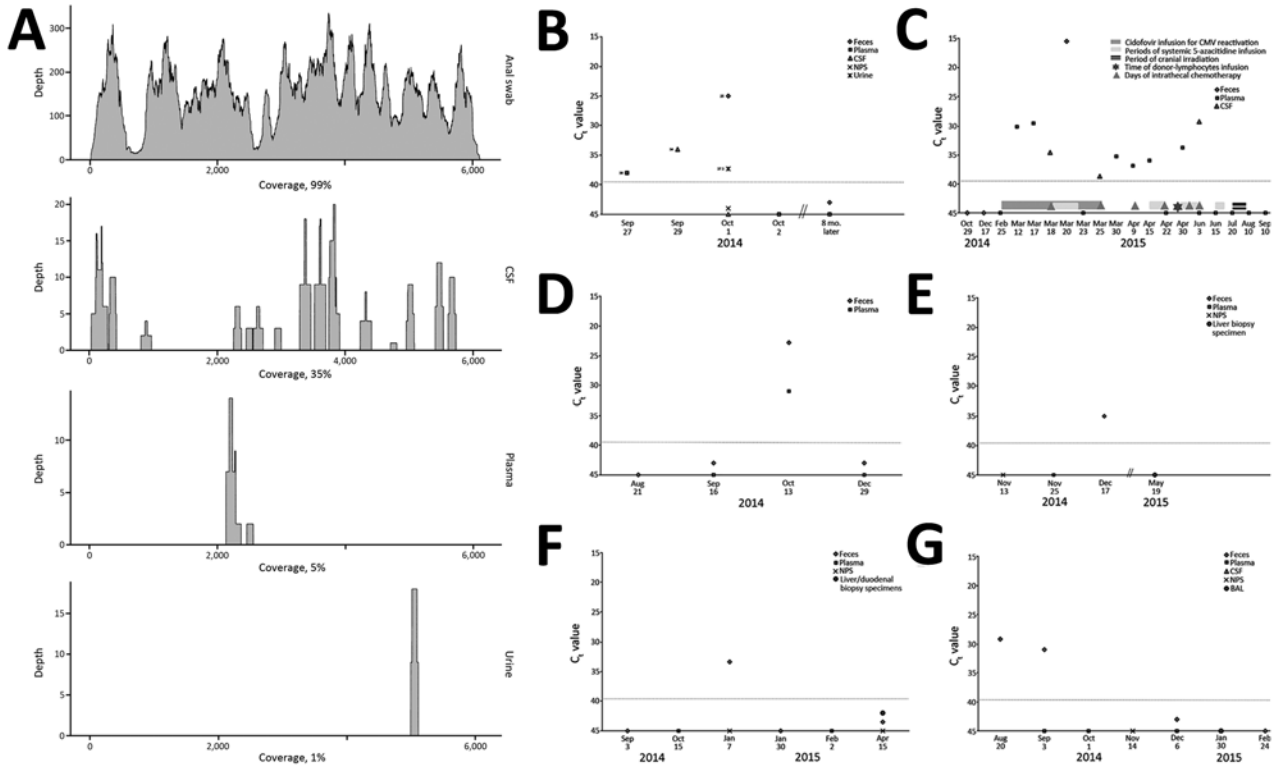

Figure 2. Details of the cases of astrovirus MLB2 infection, Geneva, Switzerland, 2014. A) Next-generation sequencing results for the case-patient. Read coverage histogram is shown for each specimen analyzed. Percentage of genome coverage is also indicated. B–C) Real-time RT-PCR analysis results for the case-patient (B) and the HSCT recipient (C); D–G) real-time RT-PCR analysis results for the solid organ transplant pediatric recipients: liver transplant (D–F) and kidney transplant (G). Dashed lines represent the limit of PCR positivity (cycle threshold 40). CSF, cerebrospinal fluid; NPS, nasopharyngeal swab; BAL, bronchoalveolar lavage.

varicella-zoster virus remained negative on the second CSF analysis, and acyclovir was discontinued. Real-time PCR results for *Streptococcus pneumoniae* and *Neisseria meningitidis* was also negative, and ceftriaxone was discontinued after 7 days. The patient continued to improve; she was discharged 10 days after admission with a presumptive diagnosis of viral meningitis.

The second patient (Figure 2, panel C) was a HSCT recipient screened by the pilot prevalence study. He was a 37-year-old man who underwent HSCT on October 2014 for acute myeloid leukemia. His household included young children. In March 2015, he experienced a headache and was ultimately given a diagnosis of a leukemic relapse, including meningeal involvement with a CSF leukocyte count of 2,240 cells/μL and a flow cytometry result confirming that 90% were blast cells. Magnetic resonance imaging showed signs of meningeal leukemic infiltration without cerebral involvement. The patient subsequently received 6 cycles of intrathecal chemotherapy and 4 cycles of 5-azacitidine, which led to remission. LPs on March 25 and June 3 revealed CSF leukocyte counts within normal limits that were nonetheless difficult to interpret given the patient's marked systemic leukopenia (0.7 and 1.1 × 10^9 cells/L, respectively). At that time, the patient experienced episodes of vertigo, limb weakness, lightheadedness, and recurrent headache, for which follow-up magnetic resonance imaging was performed. Although meningeal infiltration had diminished, it was still detectable. Thus, the patient underwent cranial irradiation beginning in July 2015. The patient then received a diagnosis of a second relapse of leukemia and died in December 2015.

Discussion

Detection of astrovirus MLB2 RNA in the CSF of the initial case-patient with acute meningitis highlights the conclusion that as-yet-unrecognized potential new human pathogens can be identified by means of molecular unbiased screening in appropriately targeted populations. The subsequent detection of the same virus's RNA in fecal specimens of 6 additional patients (of whom 5 were immunocompromised, 2 had viremia, and 1 had a positive CSF specimen) demonstrates that this virus circulates in the community and could be an unrecognized cause of

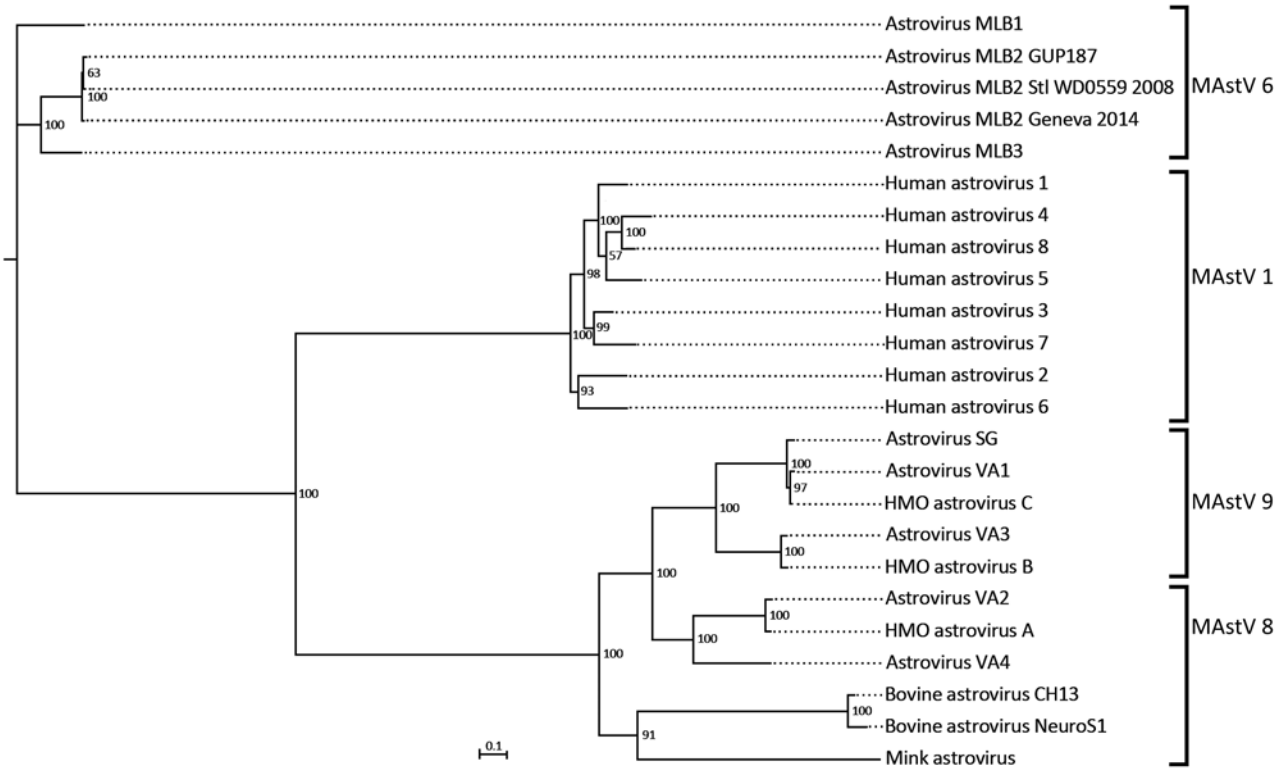

Figure 3. Phylogenetic tree constructed on the basis of full-length sequences of astroviruses and mamastroviruses. The sequence from the case-patient in this study is astrovirus MLB2 Geneva 2014. Brackets indicate the 4 Mamastrovirus species (MAstV 1, 6, 8, 9) from humans. Virus names and corresponding GenBank accession numbers are listed in online Technical Appendix 2 Table 1 (http://wwwnc.cdc.gov/EID/article/22/5/15-1807-Techapp2.pdf). Scale bar indicates nucleotide substitutions per site.

certain clinical manifestations, particularly in patients at increased risk for complications.

Although several studies have demonstrated that novel species of human astroviruses are circulating throughout the world, their associated clinical manifestations require further characterization. To the best of our knowledge, 5 cases of astrovirus CNS infection have been reported in humans, 1 caused by classic HAstV-4 and 4 caused by HAstV-VA1/HMO-C/PS (Table), but none attributed to the distant astrovirus MLB2 described here. In 2011, Wunderli et al. described a cluster of 3 children in a pediatric stem cell transplantation unit who were infected by classic HAstV-4 (*12*). Disseminated viral infection was diagnosed in 1 child who died of multiple organ failure; astrovirus was detected in several organs, including the brain and bone marrow. Similarly, HAstV-VA1/HMO-C has been detected in a few immunocompromised persons who had CNS infection and encephalitis (*13*–*16*). In animals, 2 astroviruses closely related to HAstV-VA1/HMO-C have been identified, 1 in minks with so-called shaking mink syndrome, the other in cattle with nonsuppurative encephalitis (*28*–*30*).

We have been able to partially fulfil the criteria proposed by Fredricks and Relman to show microbial disease causation on the basis of molecular tests (*31*): the high viral load observed in the fecal specimens of the 2 patients with meningitis suggests that the gastrointestinal tract is the primary site of replication, which is consistent with the tropism of this family of viruses. From the anal swab specimen of the initial case-patient, we were able to sequence the whole genome and thus demonstrate the presence of the entire virus. The transient presence of viral RNA in plasma and CSF with cycle threshold values indicating a lower viral load suggests hematogenous dissemination from the gastrointestinal tract to the meninges. With resolution of the disease, blood and fecal samples were negative for HAstV MLB2 RNA. Thus, a causal link between astrovirus MLB2 and the case-patient's acute meningitis is highly plausible. In contrast to patients in previous reports, the case-patient in our study was immunocompetent with an uncomplicated clinical course, suggesting that these viruses should probably not be considered as purely opportunistic.

In the HSCT recipient, the protracted, relatively high viral loads detected in plasma and CSF potentially mirrored

Table. Clinical cases of astrovirus infection recovered outside the digestive tract in humans by next-generation sequencing or real-time RT-PCR*

Authors (reference or figure)	Astrovirus strain	Species	Sample analyzed and results					
			Brain biopsy/CSF	Plasma/ serum	Feces	Urine	NPS	Other
Holtz et al. (*11*)	HAstV MLB2	MAstV 6	NP	+	NP	NP	+	NP
This study	HAstV MLB2	MAstV 6						
Case-patient (Figure 2, panels A, B)			+	+	+	+	–	NP
HSCT recipient (Figure 2, panel C)			+	+	+	NP	NP	NP
Patient D (Figure 2, panel D)			NP	+	+	NP	NP	NP
Wunderli et al. (*12*)	Classical HAstV serotype 4	MAstV1						
Patient 1			–/NP	+	+	–/NP	+	-/NP
Patient 2			+	+	+	–/NP	–/NP	+†
Patient 3			+	–/NP	–/NP	–/NP	+	–/NP
Quan et al. (*13*)	HAstV-PS	MAstV 9	+	NP	NP	NP	NP	–‡
Brown et al. (*14*)	HAstV-VA1/HMO-C-UK1	MAstV 9	+	+	+	NP	NP	NP
Naccache et al. (*15*)	HAstV-VA1/HMO-C-UK1	MAstV 9	+	NP	NP	NP	NP	NP
Fremond et al. (*16*)	HAstV-VA1/HMO-C-PA	MAstV 9	+	NP	NP	NP	NP	NP

*RT-PCR, reverse transcription PCR; CSF, cerebrospinal fluid; NPS, nasopharyngeal swab; HAstV, human astrovirus; MAstV, mamastrovirus; NP; not performed; +, positive; –, negative; HSCT, human stem cell transplant.
†Vesicle swab, heart, lung, spleen, bone marrow, kidney, small intestine.
‡Kidney, liver, spleen.

the cycles of immunosuppressive therapy he concomitantly received. Yet, other factors, such as his underlying illness or potential drug toxicity, could have caused and maintained his neurologic symptoms. Nonetheless, our observations indicate that astroviruses cause viremia and express CNS tropism; these findings provide a plausible explanation for the encephalitis cases recently described (*12–16*). The source of infection in these patients is unknown, although they may have been infected by contact with children. The additional detection of the virus in 5 fecal specimens from children supports this hypothesis. Alternatively, although no animal astrovirus MLB2 reservoir has yet been identified, zoonotic transmission remains another possibility (*17*,*32*).

We assessed the potential circulation of this unrecognized virus in humans by examining its prevalence in different biological specimens of interest. Our hospital-based investigation over a 1-year period found an incidence rate of astrovirus MLB2 infection of 1.1% (including the case-patient) in feces, which is higher than found in most other studies (*3*,*9*,*33*,*34*). In comparison, in our hospital, 1-year incidence rates of 3 other enteric viruses, noroviruses, rotaviruses, and enteroviruses, were 5.5%, 6.7%, and 2.7%, respectively. Whether the global prevalence of astrovirus MLB2 is underestimated or fluctuates from year to year remains to be determined. Unlike results from a previous report (*14*), CSF samples were successfully screened, with a positivity rate of 0.5% (2/405), which supports consideration of the virus in the investigations of unexplained CNS infection. Curiously, a classic symptom of human astrovirus infection is headache (*2*,*4*).

The pathophysiology and clinical manifestations of astrovirus MLB2 and other astroviruses require further definition. Of 7 patients with astrovirus MLB2 in feces, in only 1 patient did this finding have a clear clinical correlation with digestive symptoms. Thus, as with noroviruses (*35*), carriage may be prolonged after a subclinical or transient gastrointestinal illness or, as with classic astroviruses or enteroviruses, gastrointestinal replication and carriage may occur without digestive symptoms. Indeed, in a recent case-cohort study, astrovirus MLB2 was recovered in the feces of 8 patients who did not have overt digestive symptoms (*9*).

Additional virologic and epidemiologic investigations are required to assess our findings; however, seroresponses could not be evaluated because of the lack of an available antibody assay. In the absence of neural tissue sampling, in situ hybridization could not be considered. We could not isolate or demonstrate active viral replication because of the lack of a cell culture system for novel astroviruses. Furthermore, our RT-PCR assays were not quantitative, although cycle threshold values gave substantial information. These factors require more laboratory investigations, which are justified by the potential clinical effects of astroviruses that this study has highlighted. Finally, our prevalence study was retrospective and did not include healthy control patients, limiting our ability to draw solid conclusions with regard to associated disease patterns.

Although we do not provide evidence of disease causality for HAstV MLB2, according to classic Koch's postulates, our preliminary findings could place astrovirus MLB2 in the differential diagnosis not only of diarrhea but also of aseptic meningitis and protracted infection in highly

immunocompromised hosts. Potential determinants of extraintestinal dissemination, such as viral load kinetic, immune response, and host and viral genetic factors, require further characterization. Should further studies confirm our findings, patients with unexplained meningoencephalitis and those with severe immunosuppression should be considered for astrovirus MLB2 screening.

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Astrovirus MLB2, a New Gastroenteric Virus Associated with Meningitis and Disseminated Infection

Technical Appendix 1

Hôpitaux Universitaires de Genève

Laboratoire de Virologie
Service de médecine de laboratoire
Département de médecine génétique et de laboratoire
Service des maladies infectieuses
Département des spécialités de médecine
Prof. Laurent Kaiser
Secteur microbiologie

Geneva, August 25th 2015

Investigation of central nervous system/respiratory diseases of unrecognized viral etiology

A. Background

Viral nucleic acid-based identification has changed the face of clinical virology during the last decade. Screening for common viral infections by polymerase chain reaction (PCR) or reverse transcription (RT-PCR) is performed on a daily basis and, if needed, viral loads can be quantified. Although a specific virus can be targeted individually, it is also common practice to use panels containing multiple targets adapted to specific syndromes. These panels can be restricted to a handful of viruses in the case of central nervous system (CNS) infections or can target more than 17 different agents in the case of respiratory tract infections. Despite this progress, it is still common for infectious disease specialists to be faced with cases for which a viral disease is suspected, but where all microbiological investigations remain negative or incomplete. Indeed, up to 40% of encephalitis cases, presumably of infectious origin remain, of unknown etiology despite extensive microbial investigations. Although observed in a lower extent, the same situation is noted in studies investigating the cause of pneumonia or lower respiratory tract infections. This problematic is clearly demonstrated in the following table that illustrates the total number of CSF and bronchoalveolar lavages (BAL) received in our routine laboratory in 2011 and the respective positivity rate.

	CSF	BAL
Total number of cases	477	245
% Positivity	**18.2 %**	**23.2 %**

Next-generation sequencing (NGS) technologies are evolving very rapidly and have provided the most powerful tools for discovering previously unrecognized human pathogens. Originally, NGS became available in 2005 and was widely used for whole genome sequencing due to its ability to rapidly generate vast amounts of sequence information. Since then, several NGS platforms based on different biochemistry and sequencing protocols have evolved and are currently commercially available (Illumina, 454 pyrosequencing, Ion Torrent, SOLiD, etc.). Pertinent to our investigation discussed below, NGS has been used for key investigations in clinical virology enabling the discovery of several novel viruses, such as:

1. Two recently identified arenaviruses responsible for patient deaths after solid organ transplantation and hemorrhagic fever.
2. A new polyomavirus (MCPyV) associated with most Merkel cell carcinomas.
3. A new phlebovirus associated with severe febrile illness in the USA.
4. A new enterovirus genotype identified in stool samples from children with acute flaccid paralysis.
5. A novel picornavirus associated with gastroenteritis.
6. New influenza H3N2 variant virus of swine origin.
7. The very recent identification (September 2012) of a novel coronavirus resulting in acute respiratory distress syndrome or patient death in subjects that had travelled in Saudi Arabia.

B. Goal

Our study will use NGS technologies as a tool for the discovery of new virus or distant variants from specimens collected on 4 years (starting in June 2013) in the University Hospital of Geneva from adults/pediatrics patients for whom a meningo-encephalitis is diagnosed and etiology remains unknown. Although the causative agents of acute meningo-encephalitis have already been investigated in different studies, very few have conducted systematic pre-established microbiological guideline. The identification of each case in a timely manner, together with the systematic storage of biological specimens other than plasma and CSF, will increase our ability to investigate appropriately each case. Beyond the technology, our aim is to link NGS to relevant investigations in the field of clinical virology. But most importantly, this can also be done due to our access to very relevant clinical specimens in highly selected cohort of patients. In the case of identification of novel virus sequences, this study will enable to develop novel diagnostic tools, such as specific real-time (RT)-PCR assays. Similarly, we will also include patients presenting a clinical syndrome of probable infectious etiology without identified causative microorganism despite complete investigations (i.e.: pericarditis, pleural effusion, pneumonia, hepatitis, fever of unknow origin).

C. Protocol

Identification of patient, consenting of adult patient or parents/guardians and enrolment :

This investigation will include patient (adults and children) presenting to our institution with a clinical diagnosis of meningitis, meningo-encephalitis or a clinical syndrome suspected to be associated to a pathogen agent. Only patient for which the first line of microbiological investigations remain negative will be included in this study. Physician in charge will describe the purpose and the procedure of this study, possible risk/benefits, the rights and the responsibilities of participants. If the adult patient or parents/guardians agree to participate, they will be asked to sign and informed consent form (IC) except if estimated without the mental competency to understand the IC. No analysis will be performed before the signature of the IC. The study will enroll patients for a minimum of 4 years starting in June 2013. The estimated sample size over this period is 20-30 patients per year.
Before the design of the study presented here, the investigators from the Laboratory of Virology have previously identified and selected cases of meningitis, or meningo-encephalitis, as well as others clinical cases for which an infection is highly suspected but remain of unknown etiology despite extensive microbiological analysis. These specimens will be included in this study.

Study specimen collection :

Enrolment:
At enrolment the following specimen will be collected:

- Lumbar puncture will be performed in case of suspicion of meningitis/meningo-encephalitis and only for medical reasons according to the University of Geneva Hospitals guidelines and practice and will not be performed specifically for the study. When lumbar puncture is performed for normal clinical care, the non-used volume will be stored for the study purposes' if available. For pediatric patients, the maximum volume will be collected according to patient's weight, following the standard of care.

- EDTA plasma and serum collection will be performed only for medical reasons according to the University of Geneva Hospitals guidelines and practice and will not be performed specifically for the study. When EDTA plasma or serum collection is performed for normal clinical care, the non-used volume will be stored for the study purposes' if available. Optional, additional EDTA plasma and serum specimen will be collected in a separate vial for study purposes'.

- If not collected for normal clinical care, a nasopharyngeal swabs, a stool (or rectal swab) and urine specimen will be collected for study purposes'.

- Other samples such as bronchoalveolar lavages, pericardial, pleural, or any other sterile fluid might be eventually analyzed for selected cases. All these samples will be exclusively collected for medical reasons.

- Other samples such as bronchoalveolar lavages, pericardial, pleural, or any other sterile fluid might be eventually analyzed for selected cases. All these samples will be exclusively collected for medical reasons.

Samples will be used exclusively for the detection of new pathogen agents. No human genetic study will be performed.

After obtaining the patients agreement, our study requires planning two supplementary visits per patient. The first one is dedicated to collect specimen specifically for this project by the study medical doctor or nurse in case of absence of pathogen agent identification after initial screening performed by routine assays and will be done within the 24 hours after obtaining routine assays results. The second visit is optional and is planned 30 days after discharge to follow-up clinical evolution.

While hospitalized or Follow-up:
Any specimen collected for medical reasons during the hospitalization will be stored after analysis and could be used for the purpose of this study if needed. During hospitalization, blood (optional), urine, respiratory swab and stool specimen may be performed specifically for the purpose of this study after agreement of the patient.
Optional, an additional convalescent plasma specimen (EDTA tube) will be collected on the day of discharge and day 30. Patient will be asked to come back to the hospital if discharged.

Time frame:

The study will enroll patient immediately after ethical approval and will be conducts for a minimum of 4 years.

Study organization:

The study will be coordinated by investigators from the Laboratory of Virology, pediatricians in charge of the emergency division and infectious diseases unit and neurologists from the University of Geneva Hospitals. Investigator will be in charge to organize in their relative units the specimen collection mentioned above.

Laboratory of Virology	Prof Laurent Kaiser (main investigator), Dr Samuel Cordey, Dr Manuel Schibler, Lara Turin and Dr Diem-Lan Vu Cantero
Infectious diseases Service	Prof Laurent Kaiser
Pediatric Department	Prof Klara Posfay-Barbe
Pediatric emergency Division	Prof Alain Gervaix
Pediatric neurology	Dr Joël Fluss and Dr Christian Korff
Adult neurology	Prof Patrice Lalive
Adult emergency room	Olivier Rutschmann
General Medicine	To be determined

Case report form (CRF):

Patient information relevant to the viral infection of unknown etiology will be recorded on an individual CRFs (see enclosed document) designed for this study. Data will be entered on the day of enrolment and during follow-up by investigators. The information contained within the CRFs will be transferred to a computerized database and will be available exclusively to the study team.

Biological Specimen:

Original specimen collected for the purpose of this study will be stored at the appropriate temperature (70°C for CSF, plasma, nasopharyngeal swab, stool, urine specimen and other samples; -20°C for serums) within 24 hours and aliquoted as indicated in specific SOPs. Samples collected will be used for the purpose of this study as stated in the protocol and stored for future use. After specimen analysis, the non-used volumes will be stored by the investigators throughout the period dedicated to the study. Any proposed plans to use samples other than for those investigations detailed in this protocol will be submitted to the relevant ethics committees prior to any testing.

Fortuitous discovery of others viral infection:

In case of fortuitous discovery of viral infection(s), the result will be communicated to Prof. Laurent Kaiser, Dr Klara Posfay-Barbe, Prof. Patrice Lalive or Dr Joël Fluss, and an additional physician from the Infectious diseases Service from the University of Geneva Hospitals not involved in this study. This physician will evaluate the clinical relevance of the fortuitous discovery and decide to transmit or not the information to the patient's doctor. This latter will be in charge to contact directly his patient.

Additionally, in case of discovery of a pertinent and/or novel viral infection, further retrospective investigations on stored biological specimens of other patients will be performed in order to understand the virus' prevalence, pathogenesis and associated clinical syndrome. In case of positive results, clinical data of concerned patients will be reviewed by the infectious diseases specialist and potentially the medical doctor in charge. In case of publication, all data will be anonymously provided.

Specific SOP:

ENROLMENT

Potential participants are patients admitted to Emergency Department , the Intensive Care Unit (ICU), Infectious Diseases Ward or in the General Medicine, Neurology, Cardiology, Pneumology or Gastroenterology.

The medical doctor in charge and the investigators will evaluate the following screening criteria:

INCLUSION:

1. Case with clinical syndrome of probable infectious etiology
2. Absence of pathogen agent identification after initial screening performed by routine assays.
3. Informed consent given by adult patients, parent or legal guardian

The medical doctor in charge and the investigators (MD and study nurse) will discuss the study with the adult patient, parents or legal guardians of potential participants and request informed consent. Parents or legal guardians who agree will sign the IC. Once the IC signed, a dedicated CRF will be completed.

Anonymous CRF

The medical doctor in charge and the investigators will interview and examine the patient and will fill the CRF.
The medical doctor in charge or nurse will collect and store the biological samples as described below and record this on the CRF.

Specific inclusion criteria for meningitis or meningo-encephalitis:

1. Case with clinical diagnosis of meningitis or meningo-encephalitis
2. No contra-indication to perform a lumbar puncture for CSF collection
3. Absence of pathogen agent identification in CSF after initial screening performed by routine assays.
4. Informed consent given by adult patients, parent or legal guardian

Initial screening routine assays available in the laboratory for meningitis and meningo-encephalitis

Serological tests to screen for the following acute primary infections:

- EBV, CMV, HHV6, HIV, mumps, TBE, *Borrelia*.

PCR and RT-PCR assays:

- CSF: HSV1 and 2, VZV, enterovirus, parechovirus and HHV6.
- Respiratory specimens: for all known respiratory viruses (influenza, RSV, parainfluenza, coronavirus, metapneumovirus, rhinovirus, enterovirus, adenovirus, bocavirus).
- Stools: rotavirus, norovirus, astrovirus, sapovirus, enterovirus, parechovirus, and adenovirus.

The screening assays will not be taken in charge by the study fundings. **Further screening potentially performed for the study purposes** According to epidemiological factors, the following analysis will be systematically conducted: o Serology in blood: West Nile, Japanese encephalitis, Toscana. o RT-PCR: LCMV and Toscana virus in CSF. According to initial results, serological screening using CSF itself will be completed whenever needed according to recognized procedures (Lyme, VZV, HSV mainly). These additional screening will be taken in charge by the study fundings' (Annex 1).	
RESEARCH SWABS The medical doctor in charge or nurse will collect 1. A nasopharyngeal swab in viral transport medium (if none previously collected for normal clinical care). 2. A stool specimen (or rectal swab) in a stool container (if none previously collected for normal clinical care). 3. A urine specimen in appropriate vial (specifically collected for the study purposes'). The study medical doctor or nurse will complete the fields of the appropriate labels and label the samples, and will complete one row on the sample log form on the ward.	**Note:** Sample swabs, VTM, stool containers, urine vial supplied by HUG
LUMBAR PUNCTURE The medical doctor in charge and the investigators will collect cerebrospinal fluid (CSF) in case of meningitis or meningoencephalitis as needed for normal clinical care. If available, the non-used volume will be stored for the study purposes'. The study medical doctor or nurse will complete the fields of the appropriate labels and label the samples, and will complete one row on the sample log form on the ward.	**Note:** Lumbar puncture kits and tubes supplied by HUG
BLOOD SAMPLES The medical doctor in charge or nurse will collect blood samples (plasma, serum) as needed for normal clinical care. If available, the non-used volume will be stored for the study purposes'. If the plasma or serum non-used volume is < 1ml, a minimum of 1ml blood sample in an EDTA tube and a minimum of 1ml blood sample in a serum tube will be collected for the study purposes'. The study doctor or nurse will complete the fields of the Day 0 EDTA/Serum label and label the samples, and will complete one row on the sample log form on the ward.	**Note:** EDTA tubes, serum tube supplied by HUG

OTHER SAMPLES Depending on the clinical presentation, the medical doctor in charge or nurse may collect other samples as needed for normal clinical care. If available, the non-used volume will be stored for the study purposes'. Potential other samples are: • Pericardial fluid • Pleural fluid • BAL (bronchoalveolar lavage) • Solid material (i.e.: hepatic biopsy) These samples will be collected only if needed for medical indications. Specimens will not be collected only for study purposes. No additional volume will be collected for study purposes. The study medical doctor or nurse will complete the fields of the appropriate labels and label the samples, and will complete one row on the sample log form on the ward.	**Note:** Storage material supplied by HUG
STORAGE The nasopharyngeal swab, stool sample (or rectal swab), urine sample, CSF, blood and other specimens will be immediately stored at 4°C in the in the fridge. The doctor in charge has the responsibility to ensure that all collected samples and the sample log forms will be transferred to the Laboratory of Virology at the end of each working day (5 pm) and on Monday mornings (9 am) for storage at -70 °C. As soon as the Laboratory of Virology receive the samples and the sample log forms, investigators have the responsibility to aliquot (CSF = 250 ul, EDTA tube/Serum = 500ul, Nasopharyngeal swab in VTM = 1 ml, Stool = 1 ml, Urine = 1 ml) and store the samples at -70 °C or -20°C. Specimen will be kept and use only for the presented study. After specimen analysis, any leftover will be stored by the investigators. These specimens will only be used for microbiological investigations as described in our protocol or complementary virological characterization if needed. No human genetic analysis will be done.	
# DISCHARGE	
RESEARCH BLOOD Optional, the doctor in charge or nurse will take a blood sample in an EDTA tube for the study. The doctor in charge or nurse will complete the fields of the EDTA Discharge label and label the samples, and will complete one row on the sample log form on the ward.	**Note:** EDTA tubes supplied by HUG Labels and sample logs supplied by HUG
# FOLLOW-UP	
Optional, after discharge, the patients will be called by the doctor in charge or by investigators to follow-up clinical evolution. Follow-up visits will be scheduled at day 30 or thereafter if clinically indicated.	

BLOOD SAMPLES Optional, the doctor in charge or nurse will take a blood sample in an EDTA tube for the study. The doctor in charge or nurse will complete the fields of the EDTA Follow-up label and label the samples, and will complete one row on the sample log form on the ward.	**Note:** EDTA tubes supplied by HUG Labels and sample logs supplied by HUG

Additional investigations

Retrospective

Optional, extensive retrospective analysis will be performed on biological specimens retrieved for routine clinical care and stored in any laboratory of the University of Geneva Hospitals or Geneva University of medical school.

Main biological specimens include:

- Stools
- Blood
- CSF fluid
- Biopsies
- Respiratory specimens (NPS, BAL)
- Urine
- Other specimens can be analysed depending on the virus' tropism.

Analysis performed:

- Molecular diagnostic tests (PCR, NGS)
- Antigen or antibody detection
- Culture

In case of a positive result, clinical data will be reviewed by the infectious diseases specialist in charge of the investigation.

Prospective

In case of prospective additional investigation concerning other patients than the case patient issued from the initial study protocol, a separate protocol or novel amendment will be submitted to the local ethics committee.

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Laboratoire de Virologie

Service de médecine de laboratoire
Département de médecine génétique et de laboratoire

Service des maladies infectieuses
Département des spécialités de médecine
Prof. Laurent Kaiser
Secteur microbiologie

Genève, le 25 août 2015

FORMULAIRE D'INFORMATION ET DE CONSENTEMENT (version 4)

Madame, Monsieur,

Vous êtes invité à participer à une étude intitulée « ***Recherche des nouveaux agents viraux associés aux méningites, méningo-encéphalites et autres infections d'origine indéterminée***» car vous, votre enfant ou votre proche présentez une infection pour laquelle aucun microbe n'a été identifié.

Le présent document vous renseigne sur les modalités de ce projet de recherche. Pour participer à ce projet, vous devrez signer le consentement à la fin de ce document et nous vous en remettrons une copie signée et datée.

Informations générales sur l'étude clinique et son financement

Cette étude a pour objectif de mettre en évidence d'éventuels nouveaux virus associés aux infections virales. A ce jour, pour un grand nombre d'infections, il est impossible pour les laboratoires d'analyses d'identifier le microbe responsable (p.ex. virus, bactérie, champignon). Ceci s'explique par le nombre important de microbes pouvant être en cause et aussi par le fait que probablement un certain nombre de microbes restent à découvrir. Cette recherche sera réalisée via les échantillons de sang et ainsi que d'autres prélèvements effectués pour effectuer le diagnostic initial de l'infection selon les règles habituelles. Des prélèvements supplémentaires, tels que des frottis de gorge, selles ou urines pourront être spécifiquement effectués pour cette étude. Cette étude est menée au sein des différents services des HUG assistant des patients adultes ou pédiatriques.

Cette étude est financée par le Fonds National Suisse de la recherche scientifique.

Objectifs du projet

Cette étude a pour principal objectif de rechercher et identifier des nouveaux virus responsables d'infections dont l'origine reste indéterminée malgré des investigations complètes.

Déroulement de l'étude

Vous serez traité et suivi conformément à la prise en charge standard en vigueur dans notre établissement pour ces infections. Une fois votre accord donné, nous effectuerons des analyses supplémentaires sur l'ensemble des prélèvements collectés initialement pour effectuer le diagnostique de l'infection ainsi que sur les différents frottis effectués spécifiquement pour cette étude. Aucune analyse génétique sur l'ADN humain ne sera réalisée.

En cas de découverte fortuite d'une autre infection virale, les résultats seront analysés par un groupe de spécialistes en infectiologie des Hôpitaux Universitaires de Genève. Ces derniers décideront de la pertinence de cette découverte et transmettrons les résultats considérés utiles à votre médecin traitant qui se chargera si besoin de vous contacter directement.

Droit de retrait sans préjudice de la participation

Il est entendu que votre participation à ce projet de recherche est tout à fait volontaire et que vous restez libre, à tout moment, de mettre fin à votre participation sans avoir à motiver votre décision ni à subir de préjudice de quelque nature que ce soit.

Risques et désagréments

La participation à cette étude ne comporte aucun risque personnel.

Bénéfices

Votre participation pourrait permettre la détection de nouveaux microbes ou virus confirmant le diagnostic de votre infection. Ceci permettra également de développer ou d'inclure de nouveaux tests diagnostics pour la prise en charge future de patients avec une infection d'origine indéterminée.

Confidentialité

Le laboratoire de Virologie et les autres services des HUG impliqués dans l'étude garantissent la confidentialité de toutes les données en conformité avec la législation suisse sur la protection des données.

Les données du projet de recherche pourront être publiées dans des revues scientifiques. Aucune publication ou communication scientifique ne renfermera d'information permettant de vous identifier.

Indemnité

Vous ne percevrez aucune indemnité financière pour participer à cette étude.

Personnes de contact et investigateur principal du projet

En cas de questions pendant ou après l'étude, vous pouvez à tout moment contacter les responsables suivants pour l'étude: Dr. Samuel Cordey 079 553 3645 ou Dr. Manuel Schibler 079 553 4816. Pour les cas pédiatriques, merci de contacter le Prof Klara Posfay-Barbe 079 553 2586.

Pour toute question information complémentaire, l'investigateur principal de cette étude peut être contacté : Prof. Laurent Kaiser 022 372 49 92

Aspect éthique

Le protocole a été approuvé par le Chef de Service et le Comité d'Ethique de la recherche des Hôpitaux Universitaires de Genève.

Consentement du participant

Je, _____ *(nom, prénom)*, déclare avoir lu et/ou compris le présent formulaire et j'en ai reçu un exemplaire. Je comprends la nature et le motif de ma participation au projet. J'ai eu l'occasion de poser des questions auxquelles on a répondu, à ma satisfaction.
Par la présente, je consens librement de participer au projet et affirme avoir eu suffisamment de temps de réflexion pour prendre ma décision.

Date : _____ Signature du participant : _____

Si requis, consentement de la part du responsable légal ou d'un proche

Je, _____ *(nom, prénom)*, déclare avoir lu et/ou compris le présent formulaire et j'en ai reçu un exemplaire. Je comprends la nature et le motif de participation de mon enfant ou mon proche au projet. J'ai eu l'occasion de poser des questions auxquelles on a répondu, à ma satisfaction.
Par la présente, je consens librement de faire participer mon enfant ou mon proche, _____ *(nom, prénom)* de participer au projet et affirme avoir eu suffisamment de temps de réflexion pour prendre ma décision.

Lien avec le patient : _____

Date : _____ Signature: _____

Déclaration du responsable de l'obtention du consentement

Je, _____ *(nom, prénom)*, certifie avoir expliqué à la participante ou au participant intéressé(e) les termes du présent formulaire, avoir répondu aux questions qu'il ou qu'elle m'a posées à cet égard et lui avoir clairement indiqué qu'il

ou qu'elle reste, à tout moment, libre de mettre un terme à sa participation au projet de recherche décrit ci-dessus. Je m'engage à garantir le respect des objectifs de l'étude et à respecter la confidentialité.

Date : _____ Signature : _____

Déclaration de l'investigateur principale de l'étude

Je, Prof. Laurent Kaiser, investigateur principal de l'étude, déclare que les investigateurs de cette étude sommes responsables du déroulement du présent projet de recherche. Nous nous engageons à respecter les obligations énoncées dans ce document.

Signature du chercheur principal de l'étude : _____

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Astrovirus MLB2, a New Gastroenteric Virus Associated with Meningitis and Disseminated Infection

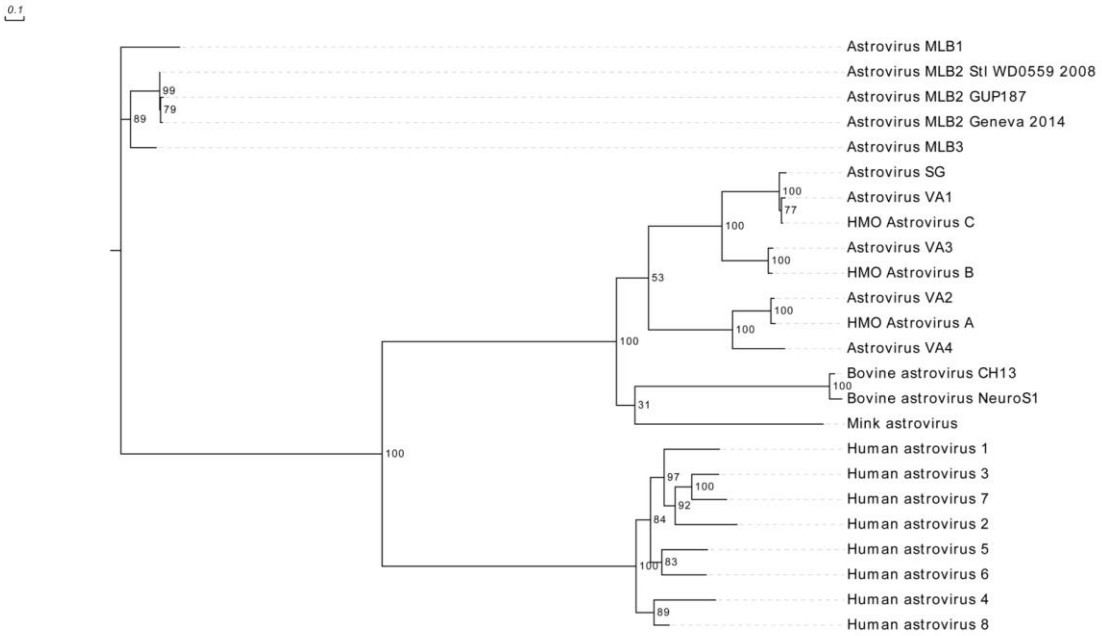
Technical Appendix

Technical Appendix 2 Table 1. Astrovirus names and GenBank accession numbers used for full-length sequences phylogenetic tree

Virus name	Accession no.	GI
Astrovirus MLB1	JQ086552.1	GI:380467967
Astrovirus MLB2 Stl WD0559 2008	JF742759.1	GI:354805828
Astrovirus MLB2 GUP187	AB829252.1	GI:523453979
Astrovirus MLB3	NC_019028.1	GI:410428374
Astrovirus SG	GQ891990.1	GI:296932862
Astrovirus VA	NC_013060.1	GI:255357299
Astrovirus VA2	NC_018669.1	GI:407868423
Astrovirus VA3	NC_019026.1	GI:410428372
Astrovirus VA4	NC_019027.1	GI:410428373
Bovine astrovirus CH13	NC_024498.1	GI:667714408
Bovine astrovirus NeuroS1	KF233994.1	GI:514389190
HMO astrovirus A	NC_013443.1	GI:262166845
HMO astrovirus B	GQ415661.1	GI:261597212
HMO astrovirus C	GQ415662.1	GI:261597215
Human astrovirus 1	KF211475.1	GI:548797282
Human astrovirus 2	KF039911.1	GI:542717312
Human astrovirus 3	GU732187.1	GI:291508554
Human astrovirus 4	KF039913.1	GI:542717348
Human astrovirus 5	JQ403108.1	GI:380846546
Human astrovirus 6	GQ495608.1	GI:259121926
Human astrovirus 7	AF248738.2	GI:14572176
Human astrovirus 8	AF260508.1	GI:9230736
Mink astrovirus	NC_004579.1	GI:28867239

Technical Appendix Table 2. Astrovirus names and GenBank accession numbers used for capsid sequences phylogenetic tree

Virus name	Accession no.	GI
Astrovirus MLB1	AFD61563.1	GI:380467970
Astrovirus MLB2 Stl WD0559 2008	AER41414.1	GI:354805831
Astrovirus MLB2 GUP187	BAN62843.1	GI:523453982
Astrovirus MLB3	YP_006905854.1	GI:410493725
Astrovirus SG	ADH93577.1	GI:296932865
Astrovirus VA	YP_003090287.1	GI:255357301
Astrovirus VA2	YP_006792628.1	GI:407868426
Astrovirus VA3	YP_006905860.1	GI:410493719
Astrovirus VA4	YP_006905857.1	GI:410493722
Bovine astrovirus CH13	YP_009047248.1	GI:667714411
Bovine astrovirus NeuroS1	AGO50636.1	GI:514389192
HMO astrovirus A	YP_003275953.1	GI:262166848
HMO astrovirus B	ACX85474.1	GI:261597214
HMO astrovirus C	ACX85476.1	GI:261597217
Human astrovirus 1	AGX15185.1	GI:548797285
Human astrovirus 2	AGV40897.1	GI:542717313
Human astrovirus 3	ADE09295.1	GI:291508557
Human astrovirus 4	AGV40905.1	GI:542717351
Human astrovirus 5	AFE84778.1	GI:380846548
Human astrovirus 6	ACV92107.1	GI:259121929
Human astrovirus 7	AAK31913.1	GI:13603731
Human astrovirus 8	AAF85964.1	GI:9230739
Mink astrovirus	NP_795336.1	GI:28867242



Technical Appendix 2 Figure. Phylogenetic tree of astroviruses based on capsid sequences. (Technical Appendix 2 Table 2 shows correspondence between virus name and accession numbers.)